Zone Electrophoresis in Columns and Adsorption Chromatography on Ionic Cellulose Derivatives as Methods for Peptide and Protein Fractionations

Application to the Study of Posterior Pituitary Hormones

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JERKER PORATH

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INTRODUCTION

This paper includes a brief summary of a number of previous reports (1–6) concerning zone electrophoresis in powder columns and new cellulose ion exchangers. It also includes additional aspects of importance for these methods.

The isolation of a melanocyte stimulating hormone (β-MSH) was achieved by zone electrophoresis with a technique developed by the author and his collaborators (7). The present work is intended to be an introduction to more comprehensive investigations on peptide hormones of the hypothalamo-hypophysial system. In illustration of the methods some exploratory experiments on purification of porcine posterior pituitary extract will therefore be described.

As our knowledge of natural products of primary biochemical importance increases we have to face more and more intricate separation problems. It is suggestive that in many cases the isolation very often is a matter of work extending over many years whereas determination of structure and synthesis of the isolated compounds nowadays sometimes may be accomplished in a surprisingly short time. Physical methods especially X-ray analysis are extremely valuable for peptide and protein investigations but no complete elucidation of the structure of a peptide or a protein can as yet be made without cleavage of peptide bonds followed by isolation and identification of split products or their derivatives. This fact also strongly emphasizes the paramount importance of efficient and reliable fractionation methods in peptide and protein research today. Thus such methods do not only play an extremely important part in isolation but also in structure determination.

Liquid–liquid counter-current extraction methods have been extensively used in purification of peptides such as, for instance posterior pituitary hormones. There is little doubt about their usefulness but unfortunately the number of applicable phase systems are rather limited. Instead of using this method—or any other method—exclusively, a combination of several procedures depending on different physical or chemical properties will, as a rule, prove to be more efficient. Among such methods the author has chosen to study adsorption chromatography on cellulose derivatives and electrophoresis. This choice fits quite naturally into the program of the Institute and is also based on the author's opinion that these methods will be of increasing importance in future work in the chemistry of peptides, proteins, nucleotides, nucleic acids and similar compounds. It is therefore necessary to work out more effective

isolation methods. This consideration has guided the author in his choice of the subject to be presented here.

Chemists working along classical lines often do not appreciate fractionation work but look upon it as a nuisance that must be passed before more interesting problems can be attacked. Frequently however, intricate isolation problems present features no less fascinating and challenging than those of other branches of chemical research.

METHODS

Zone Electrophoresis

A. *Principle of the methods*

The well known moving boundary electrophoresis apparatus as designed by Tiselius, Longsworth, Svensson and others was originally constructed as an analytical tool. It was so effective that attempts were soon made to employ slightly modified apparatus for preparative purposes (8).

In preparative electrophoresis the aim is complete separation. This means that the solution to be fractionated should be introduced into the separation cell as a narrow band or zone. Under influence of an electric field, the substances in the initial zone will migrate into the surrounding buffer with a speed characteristic for each substance. The migration of a substance is closely related to its mobility, a physical property defined for boundary electrophoresis conditions. The original zone is thus split into a number of migrating zones, corresponding to boundaries in moving boundary electrophoresis (Fig. 1).

Concentration gradients are formed which in "free" solution would cause convection and hence spoil the electrophoretic separation. Stabilization of the liquid may be achieved by use of a capillary system (consisting for instance of powder and liquid). The gain in reduced convection is, however, accompanied by the introduction of new complications. The substances to be fractionated are influenced by the presence of the "support" or "stabilizing medium".

The difficulties in finding suitable stabilizing media have delayed advances in zone electrophoresis. However, discontinuous and continuous zone electrophoresis methods have recently been developed and employed as useful tools in protein chemistry (9–15).

Convection can also be suppressed by use of density gradients (16–19). Unfortunately limited solubility of electrolytes or sample substances in the solvent making up the gradient often exclude the use of this principle.

It should also be mentioned that Kirkwood, Cann and coworkers have used controlled convection superimposed on electrophoresis for separation of polyelectrolytes (20, 21).

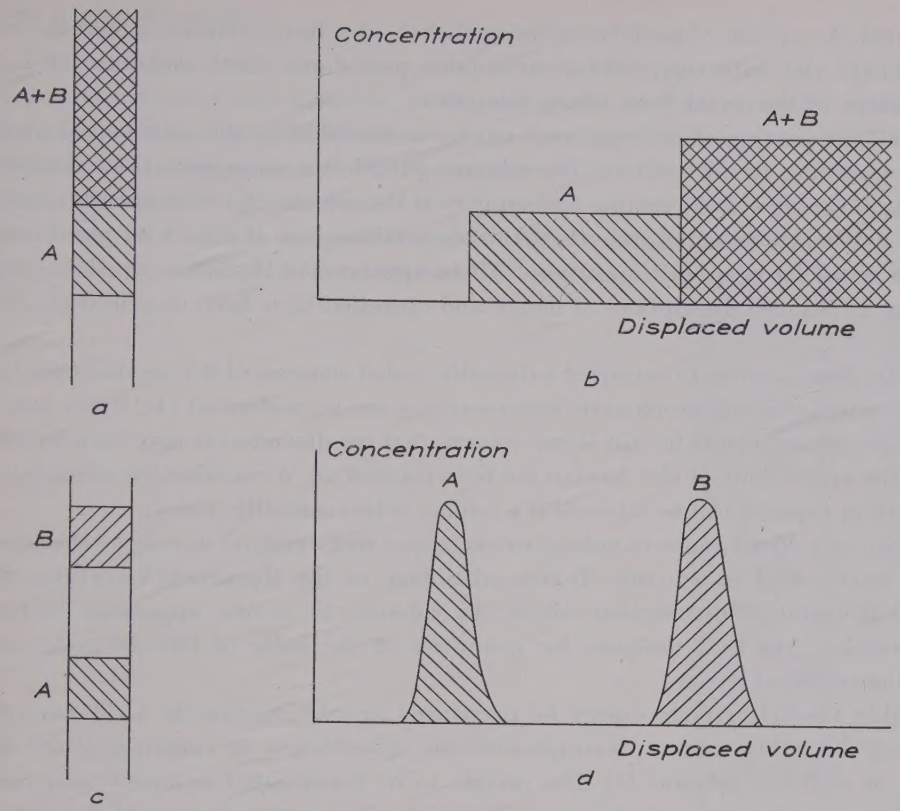


Fig. 1. The distribution of two components, *A* and *B*, on a column after moving boundary and zone electrophoresis, respectively, are illustrated in *a* and *c*. The corresponding electropherograms as obtained after determination of the distribution of the material in the solution displaced from the column are illustrated in *b* and *d*.
In boundary electrophoresis only partial resolution is attained, whereas complete separation of the components in a mixture is attempted in zone electrophoresis. The two principles are analogous to displacement and elution chromatography with respect to fractionation.

B. *Some recent studies on zone electrophoresis in powder-stabilized columns*

APPARATUS AND TECHNIQUE

Preparative electrophoresis can be performed in columns or troughs. Column procedures are more versatile than trough operations. Packing, application of material, liquid content, etc., are more easily controlled. Also liquid flow for compensation of electrophoretic migration can more conveniently be regulated especially when the hydraulic resistance is high (1).

After electrophoresis the material may be displaced from the column (22). Such a procedure has been critisized by Kunkel (10). Some of his arguments cannot be

disputed. As a result of methodological studies (1, 5) it has nevertheless been shown possible to elaborate very satisfactory column procedures which make use of displacement of the liquid from intact columns.

In all but one type of electrophoresis apparatus described by the author separation takes place in a vertical column. The columns differ in the arrangements for connection to electrode vessels, cooling and support of the column. All-glass apparatus are more convenient for small scale (1, 23), while combinations of glass with plastic are better suited for large scale operations (5). An apparatus of the latter type is shown in Fig. 2. Detailed descriptions of design and operation have been described earlier (5).

It has been possible to construct externally cooled columns of 6.5 cm diameter in which satisfactory electrophoretic fractionations can be performed (1). From temperature measurements in still larger column (8.0 cm diameter) it may be inferred that the upper limit of size has not yet been reached (5). A considerable increase in separation capacity can be achieved if a column is also centrally cooled.

So far only 30–40 grams of natural extracts (pea root exudate) have been successfully fractionated in one run. Taking advantage of the theoretical knowledge of radial temperature distribution within the columns (5) a new apparatus, under construction, has been designed for quantities of the order of 100–500 grams or 1–2 liters of blood serum.

Highly viscous extracts cannot be introduced in long columns to yield narrow starting zones, but column electrophoresis can nevertheless be employed if use is made of sectional columns (3). The sample to be fractionated is mixed with the stabilizing powder and packed in a small section which is then connected to one or two other sections containing stabilized buffer solution. Electrophoresis in the composite column may then take place in horisontal or vertical position. Only relatively crude fractionations can be performed in such columns, but they may be very useful for removing substances which would interfere in the following, more efficient procedures. Rough fractionations into acidic, neutral and basic fractions can be made conveniently.

The temperature rise within cellulose or starch columns of different dimensions may be calculated from the current and the conductivity of the electrolyte solution (5). The optimum current density in cellulose columns is unknown, but good fractionations have been achieved with radial temperature differences as high as 8–9°C.

Slow moving components in a mixture can be effectively separated on a cellulose column if their mobilities as determined by boundary electrophoresis differ by the order of 10^{-6} cm^2 $volt^{-1}$ sec^{-1} (24). It is also possible to extend electrophoresis of fastmoving components over a very long time by use of liquid flow counter-current to the electromigration (1, 5).

The capillary system has a favorable effect in two respects, it very effectively supresses *convection* and *diffusion*. Unfortunately the number of possible systems is limited since most powder substances have less desirable properties which would disturb their performance of the electrophoresis and the subsequent elution. The most important complications are: strong adsorption, low liquid content, too high resistance to flow and too great a spreading effect during electrophoresis and elution, and low stability. We shall restrict the following discussion to cellulose, starch

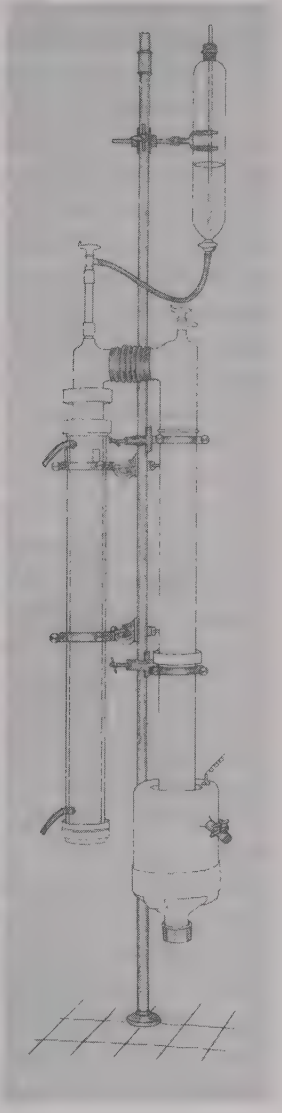


Fig. 2. Schematic drawing of an electrophoresis apparatus designed for fractionation of 100–300 ml samples ("ten gram scale"). The left-hand limb of the inverted U-tube consists of the column with cooling jacket and top pieces. Between the head and the column is inserted a short cylindrical shell which supports a central cooling tube extending throughout the whole length of the column. The liquid connection between the limbs is made possible by a special piston device at the top of the left-hand column.
During electrophoresis two electrode vessels with platinum electrodes are placed at the lower end of the two limbs (the electrode vessel under the column is removed in the Figure). The electrolyte solution is continuously circulated between the electrode vessels when long time runs are performed. The head of the column is connected to a Mariotte flask when the liquid is displaced from the columns.

and glass beads, the stabilizers that have been most extensively studied in this Institute.

(*a*) *Adsorption.*—Now and then adsorption in addition to electrophoresis may contribute to the separation but as a rule a combination of the effects is undesirable and may even prohibit use of zone electrophoresis. Strain suggests that the term electrochromatography should be used when adsorption effects are superimposed upon the pure electrophoretic separation. Separate experiments are needed to demonstrate whether chromatography contributes to the fractionation. Thus the author has shown that neutral unsubstituted amino acids are not separated from each other by chromatography on a cellulose column with 1 *M* acetic acid as solvent (1). ε-DNP-lysine, however, moves at a slower rate on the same column. Absence of reversible adsorption for such compounds is not exceptional, it is the rule on cellulose columns. In the author's opinion therefore the term zone electrophoresis, suggested by Tiselius, should be preferred when peptides or proteins are separated in an electric field with cellulose powder as support. Only when it is desirable to stress the fact that, in a certain case, the success of fractionation depends on adsorption processes should the term electrochromatography be employed. Smithies has reported extremely sharp fractionations of serum proteins in starch gel (25). No doubt this exemplifies separations achieved by electrophoresis in combination with chromatography.

As far as adsorption is concerned ethanolysed cellulose (26) generally comes closest to the ideal support for proteins and peptides. It has been pointed out, however, that irreversible adsorption of certain proteins sometimes also occurs on this support (27). Irreversible adsorption is said to occur when adsorbed substances can not be eluted by the buffer used in the electrophoresis in quantities sufficiently large to be detected by routine analysis. It is expedient to remove such substances from tissue extracts, exudates etc. as they are likely to disturb the performance of the electrophoresis. Filtration of the solution through a short column with the same medium as the one to be used in the subsequent electrophoresis will usually be sufficient (1, 27).

Many colored triphenylmethane derivatives, azodyes and other dyestuffs are adsorbed on starch as well as cellulose (1), yielding illdefined trailing if not remaining immobile at the top of the column. In such cases glass beads may be used with more success.

Since our interest, at present, chiefly concerns the electrophoretic fractionation of proteins and peptides, we will make some comments regarding possible reasons for their adsorption to cellulose. Cellulose, native or bleached, contains small amounts of carboxyl groups. For this reason cellulose is an ion exchanger although weaker than starch. It contains potential carbonyl groups which might act as weak adsorption centers or enhance the acidity of vicinal carboxyls. Furthermore, commercial cellulose always contains metal ions, hemicelluloses, lignous compounds etc. (26).

Such impurities may very likely form insoluble complexes with proteins and large peptides.

The favorable adsorption properties of cellulose ethanolysed according to Flodin and Kupke may partly be due to the low content of free carboxyl groups. The boiling in ethanolic hydrogen chloride solution causes efficient extraction of impurities, the removal of which probably is a prerequisite for the production of powder with sufficiently low adsorption.

The use of mobility differences in an electric field as a means for fractionation is of course not restricted to water as liquid medium. Mixed polar solvents containing water as one of the components are likely to be useful for electrophoresis of peptides. It should be born in mind, however, that in mixed solvents partition of solutes between two liquid phases may take place.

(*b*) *Liquid content, convection depression and heat conductance.*—It is desirable that the stabilizing medium should effectively depress convection currents without occupying too much space (28). Many gels, agar gel for instance, fulfill these conditions. On a volume basis a smaller amount of liquid is immobilized by cellulose powder although a column packed by sedimentation contains approximately 85 per cent water. The volume ratio of liquid to supporting material is much less favorable in glass powder columns. The fluid occupies the minor portion of the column (about 40 per cent). In starch water is partly free and partly bound. The content of free and bound water together makes up about 50 per cent of the column volume (28), while about 33 per cent of the water is in free form (29).

The column content may be looked upon as a plastic fluid of greater heat transmittance than the free solution (5). In cellulose and starch, the heat dissipation within the column may, at least formally, be accounted for as being effected by heat conduction only (5). This fact indicates, that convection plays a negligible role in electrophoresis. As a convection depressor, glass powder is inferior to starch as well as to cellulose.

(*c*) *Diffusion characteristics.*—A powder system favorably affects the conditions for zone electrophoresis in still another way: The grains act as barriers that reduce the diffusion. Size, channel and network structure are factors of decisive importance. Hydration of both the supporting medium and the dissolved substances results in better stabilization and more efficient depression of diffusion.

The net electrical transport is not greatly influenced by the powder. Thus in principle it should be more favorable to perform prolonged electrophoresis in a column stabilized by a capillary system, such as cellulose powder, than in density gradients.

Hitherto only few experiments have been performed in cellulose columns, packed as for electrophoresis to obtain information concerning the role of diffusion in spreading of immobile or migrating zones. It has been found, however, that the time-dependent dispersion of zones in cellulose columns is surprisingly small even for low molecular weight compounds (1). Furthermore, no significant difference in eluate

volumes as observed when comparing two similar albumin zones emerging from the same cellulose column when one of the zones had remained on the column for 14 days and the other was immediately eluted (30).

This implies that it should be feasible to extend electrophoresis over very long periods of time—probably several weeks of months. A proper choice of stabilizer with respect to its diffusion depressing properties is very important for successful long run electrophoresis.

(*d*) *Stability and rate of flow.*—Cellulose hydrolysed in boiling ethanolic hydrochloric acid solution according to Flodin and Kupke (26) for only $\frac{1}{2}$–1 hour contains only very small amounts of soluble material. (This is also true for Munktell cellulose powder.) The gain in better adsorption properties on extended ethanolysis is obtained at the expense of higher solubility. The more extensively degraded cellulose has a greater tendency to peptize and hence to clog columns, whereas the powder obtained after mild ethanolysis may be used repeatedly for half a year or more without detoriation and slowing down of the flow rate.

Starch yields a lower rate of flow than a good cellulose powder and cannot be freed so easily from soluble materials.

Glass powder is for all practical purposes completely insoluble in the electrolyte solutions used in electrophoresis. It is also insoluble in hot concentrated inorganic acids. Boiling perchloric acid can thus be used to completely remove adsorbed organic substances. Therefore, with regard to solubility glass is far superior to starch and cellulose. Rate of flow in a glass powder bed can also easily be controlled.

(*e*) *Some other properties.*—The powder must be easy to pack. Although glass powder and starch are superior to cellulose in this respect it is not difficult to pack cellulose properly. In well-packed columns the zone-spreading is within wide limits independent of the flow rate (1). It has thus been found possible to displace all the liquid in a 3 meter long column within 8–10 hours without undue spreading of the zones.

Each cellulose grain is a porous body containing channels and grooves of varying dimensions, structure and orientation. The rate of flow at a given point in the column will naturally be dependent on these variables, the resulting variation in the vertical flow vector through a given column section causes spreading of the zones. For similar reasons an electric field across the column must be microheterogeneous in nature. The micro and submicroscopic structure of the cellulose powder is therefore intimately connected to zone spreading both during electrophoresis and elution. The author has found that the spreading of a zone migrating through a certain distance during elution is very nearly the same as during the electrophoresis (1). Only in unevenly packed columns, especially in the presence of rifts will the spreading or distortion be greater during elution.

C. *Electrolyte systems*

All buffers usually employed in electrophoretic analyses of protein mixtures by the moving boundary method can also be used in zone electrophoresis in cellulose. After electrophoresis the buffer salts and the proteins can easily be separated by dialysis. Separation of inorganic salts from other dialysable ionic compounds such as amino acids, peptides, nucleotides without great losses often presents an extremely difficult problem. Electrolytic removal of buffer ions as suggested by Consden *et al.* (31) has limited application since electrode products or electrode reactions themselves cause oxidative or reductive destruction of many substances. Separation on ion exchangers is more satisfactory and has frequently been employed. As has been suggested by the author charcoal may also be used, since inorganic ions are not adsorbed. By proper pretreatment of the charcoal irreversible adsorption of the substances under investigation is reduced (32). Extraction of peptides from aqueous solution may also be achieved by organic solvents such as phenol. For application of any one of the methods mentioned satisfactory conditions must be worked out for each particular mixture. The removal of inorganic ions from a great number of samples will therefore often cause more trouble than the preceeding electrophoretic fractionation.

When dealing with dialysable substances use of inorganic and other not easily removable salts should apparently be avoided, if possible. Volatile buffers may be employed except in cases where a nonvolatile buffer plays an important role for the electrophoretic separation itself, e.g. in functioning as a complexing agent.

Aqueous solutions of acetic, formic and hydrochloric acids or mixtures of them, can be used as electrolytes of low pH. At the other end of the pH scale bases such as ammonia and organic amines may be used. Buffers of intermediate pH can be obtained by mixing weak volatile acids with volatile bases. Suitable for such mixtures are formic, acetic and carbonic acids. Ammonia, aliphatic amines, pyridine and collidine furnish the cations of volatile buffer systems. Some merits and disadvantages of the buffers have been previously pointed out by the author (2).

Pyridinium acetate and formate can very easily be evaporated *in vacuo* together with water, when in as high a concentration as 1 M or even more. Triethylammonium acetate and formate solutions are easily lyophilized in a good vacuum provided the triethylamine concentration is low (about 0.05 *M* or less). Lyophilization at higher concentrations should be performed with a strong acid as absorbent. Concentrated sulphuric or phosphoric acid may serve as good absorbents but, according to Herbertsson (of this Institute), phosphorus pentoxide is still better. Such absorbents should also be used when triethylammonium carbonate solutions are lyophilized in order to prevent shifting of pH towards extremely alkaline values. Trimethylammounium carbonate solutions can easily be lyophilized without any complications.

Adsorption Chromatography on Cellulose Ion Exchangers

Because of their hydrophilic nature, insoluble polyhydric alcohol polymers presumably provide better matrices than cross-linked polystyrene for ion exchangers to be used for the chromatographic separation of large peptides, proteins, polynucleotides and nucleic acids. The best known material of this kind is cellulose.

Ionic cellulose derivatives useful for peptide and protein chromatography

Ionic cellulose derivatives have only recently been utilized as adsorbents for peptides and proteins. Disregarding some earlier attempts of limited success, the first remarkable achievement in this field was announced in 1950 by Astwood *et al.* (33). They were able to achieve 30–50 times purification of the adrenocorticotropic hormone of the anterior lobe of the pituitary gland by use of oxycellulose. In 1956 Peterson and Sober (34) described the preparation of some cellulose ion exchangers and the use of diethylaminoethyl cellulose (DEAE-cellulose) to fractionate blood serum. Recently preparations of some other ionic cellulose derivatives were reported from this Institute (4). Thus the quaternary cellulose ether triethylaminoethyl cellulose (TEAE-cellulose) of good physical properties was obtained by ethylation of DEAE-cellulose. The preparation of two cellulose ion exchangers of the sulfonic acid type, sulfomethyl and sulfoethyl cellulose, deserves greater interest, however. Surprisingly enough SM-cellulose was synthesized for the first time. Only a short note of an unsuccessful attempt to accomplish this synthesis is known to the author (35). Such a failure is easily comprehensible because an unusually drastic treatment of the reaction mixture is necessary to bring about the etherification of alkaline cellulose with chloromethane sulfonate.

Carboxymethyl cellulose and phosphorylated cellulose should also be mentioned as examples of useful adsorbents.

STABILITY AND CAPACITY

Introduction of a large number of ionic groups into the cellulose molecules will cause gelatinization and finally solubilization of the cellulose derivatives in aqueous solutions. Thus only 0.4–0.5 milligram equivalents of sulfonic groups per gram dry powder may be introduced if good packing properties and sufficiently low solubility are to be maintained (4). DEAE- and TEAE-cellulose of higher ionic group content may be used (1.0–1.2 meq/g), but the author prefers lower capacity (0.4–0.8 meq/g) in chromatographic work, since such exchangers show less tendency to clog.

Kiessling in this Institute has shown that it is possible to increase the capacity of SM-cellulose and still retain the insoluble character by proper cross-linking of the cellulose chains. The products so far prepared deteriorate gradually in water solutions especially at low pH. It is hoped that improvements will make production of

cellulose ion exchangers of high capacity for cations possible. It should be born in mind, however, that a high degree of cross-linking and substitution may be undesirable for protein chromatography.

Anionic cellulose ethers may be regenerated repeatedly over the free form without appreciable change in physical properties. In this respect the anionic ethers are superior to strong cationic exchangers. Often repeated cyclic operations involving complete conversion to the hydrogen form of SM- or SE-cellulose will presumably cause a gradual hydrolytic degradation of the cellulose chains and consequently also production of increasing amounts of soluble material. Washing with strong inorganic acids is necessary for the complete exchange of hydrogen ions for metal or other cations. Fortunately experience has shown that if exposure to hydrochloric acid is not unduly protracted a SM-cellulose bed may be regenerated as many times as 20 or 30 and still be suitable for use. If the rate of flow decreases too much, the powder should be resuspended in a large volume of water or buffer solution, the fine grains removed and the sediment saved. The column which is packed anew will show a slightly diminished capacity than before, but a much faster flow rate.

If the conversion to the sulfonic acid form is not required acid treatment may be omitted to advantage and be replaced by prolonged washing with weak buffer solution.

Strong cellulose ion exchangers although having rather low capacity for small ions will, in the absence of salts, adsorb very large amounts of certain proteins. In the presence of large quantities of salts, desalting should therefore precede chromatography or batch operations.

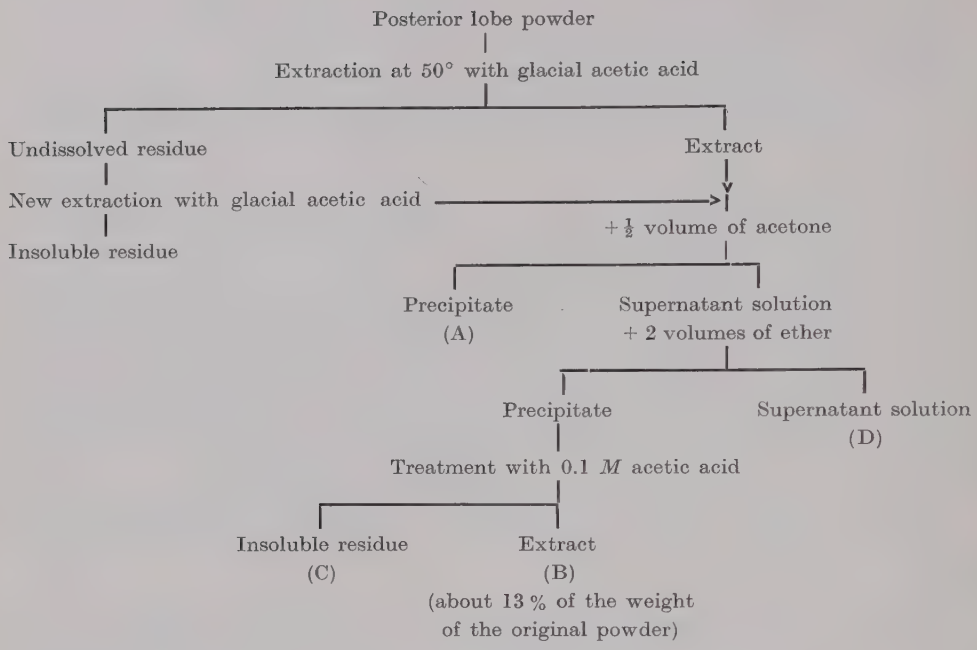
FACTORS OF IMPORTANCE FOR ADSORPTION OF PEPTIDES AND PROTEINS

The adsorption of a peptide or a protein is conditioned by the nature, number and distribution of adsorption-active centers in the molecules and the adsorbent. Carboxyl groups of the solute are attracted by basic groups of an anion exchanger, while amino, imino, imidazolyl and guanidyl groups are adsorbed by the acidic groups of a cation exchanger. Already the presence of a greater variety of basic groups suggests that cationic exchangers might possess a greater selectivity towards peptides and proteins than anionic exchangers.

From the great difference in the acidity of carboxylic and sulfonic groups it may be inferred that CM- and SM-cellulose should have widely different adsorption characteristics. This is demonstrated by the fact that neutral amino acids can be adsorbed on SM-cellulose in free form but not on free CM-cellulose. CM-cellulose may nevertheless be useful for peptide or protein mixtures as a complement to SM-cellulose. It may, for instance, be advantageous to remove strongly basic materials from a mixture before the application of the stronger ion exchanger.

Anionic exchangers like DEAE- or TEAE-cellulose in free form do not adsorb

CHART I



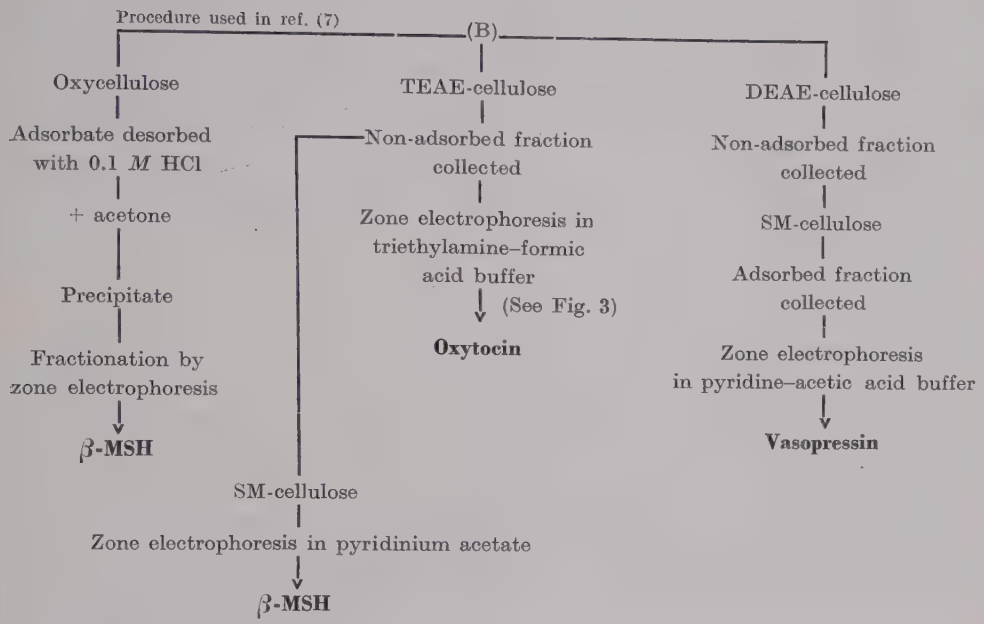
neutral amino acids to any appreciable extent. This implies that they may not be as versatile as the sulfonic exchangers. Since the adsorption-active centers of the adsorptives are different, the two types of exchangers are complementary in principle. Both types of strong ion exchangers may adsorb acidic as well as basic peptides or proteins but the order of elution of substances in a chromatogram often roughly reflects their order of isoelectric points. It is not yet known whether TEAE-cellulose has any advantage over the tertiary amine derivative DEAE-cellulose.

Other properties of importance for adsorption chromatography have been discussed elsewhere (34, 4).

ISOLATION OF PEPTIDE HORMONES FROM PORCINE POSTERIOR PITUITARY

The hypothalamus is known to produce oxytocin, vasopressin and a corticotropin releasing factor. Other not yet identified hormones may also be synthesized in this region of the brain. The hormones are transported, probably as lipid complexes, in the nerve axons through the hypophyseal stalk to the posterior lobe of the pituitary. The latter which chiefly consists of nerve tissue appears to function as a storage organ from which the hormones are released (36).

CHART II



The hormonal activities are found to be associated with material of different properties depending on the method of extraction. Thus gentle extraction procedures tend to yield active high molecular weight complexes, while after drastic treatment such as that involving trituration with warm glacial acetic acid active polypeptides can be isolated (37–39).

When preparing large batches of pituitary material it is often difficult to separate the intermediate lobe from the posterior. Therefore "posterior lobe pituitary powder" also contains substances of the intermediate lobe, e.g. MSH ("intermedin"). Moreover small quantities of anterior lobe substances may also be present in such powder. In spite of incomplete separation anatomical dissection of the glands greatly facilitates the chemical purification of the hormones.

A number of methods for purification of oxyotcin, vasopressin and melanocyte stimulating hormones are described in the literature. Unfortunately most of these methods are very complicated and their application requires much labor. The author has tried to elaborate simpler methods which should presumably be applicable to fractionation on large as well as on small scale (6).

After a crude fractionation involving extraction with warm acetic acid and precipitation with acetone and ether, a peptide concentrate is obtained (40). This concentrate contains the major part of all known posterior pituitary hormones. By use of cellulose ion exchangers and zone electrophoresis oxytocin, vasopressin and

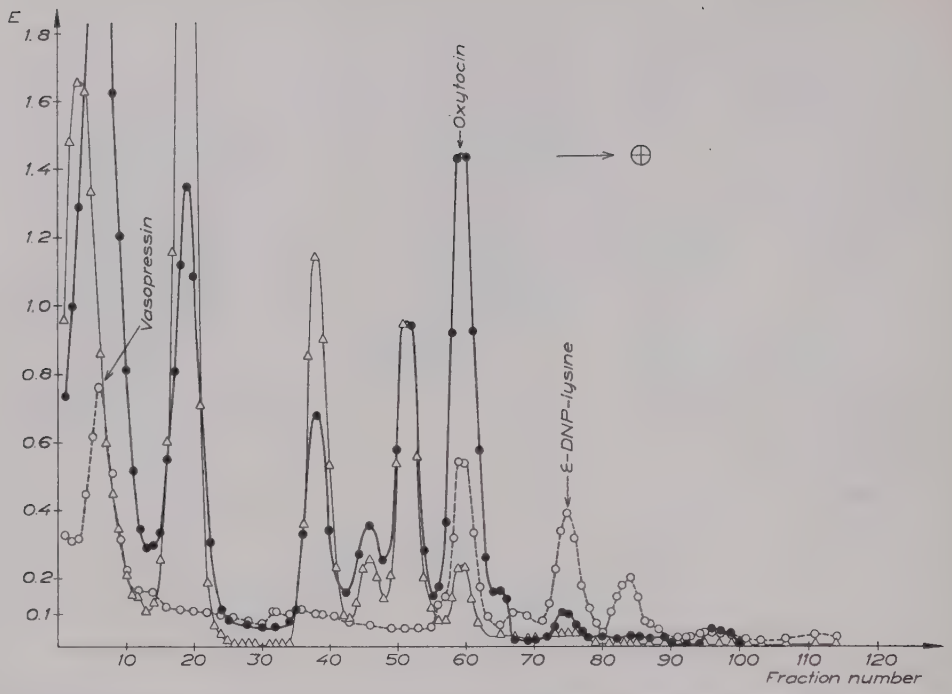


Fig. 3. Electropherogram of 160 mg of a hormone concentrate (6) (see also Chart II). The run was made on a 1.5 × 300 cm column in a triethylamine–formic acid buffer of pH 2.35 for 123 hours with a current of 20.5 mA (2800 V). ε-DNP-lysine was employed as a marker. The direction of current is indicated by the symbol →⊕. Fractions of 5 ml each were collected. –●–●–●–, ninhydrin curve obtained on hydrolysates of 0.1 ml; △—△—△, ninhydrin curve obtained directly on 0.1 ml aliquots; –○– –○– –○–, UV-absorption curve (280 mμ).

β-MSH can easily be separated. The fractionation steps are summarized in chart I and II.

Some general conclusions may be drawn from the experience obtained from these fractionations and other experiments made by the author and his collaborators:

1. Suitable conditions can more easily be found for zone electrophoresis than for chromatography. This is especially the case for proteins and large polypeptides. Paper electrophoresis provides an extremely valuable tool for finding suitable buffers for column electrophoresis. Electrophoretic runs in columns of different dimensions, on a large or a small scale, can easily be performed so that essentially the same kind of material distribution is obtained in all electropherograms. The migration of various compounds in an electrophoresis column is thus almost independent of other substances present in the original mixture except when complexes are formed or substances of the sample significantly alter the mobility determining properties of the electrolyte solution.

2. In preparative chromatography, where a high load is used, the conditions for adsorption and desorption depend not only upon the ratio between the amount of adsorbent and the mixture to be fractionated but also on the relative amounts of the components in the latter. If closely related compounds are to be separated the slow attainment of equilibrium makes a careful control of the flow rate exceedingly important.

3. By combination of chromatography on cellulose ion exchangers and zone electrophoresis in columns extremely efficient fractionation of peptide mixtures can be obtained.

SUMMARY

1. Some fractionation methods improved or introduced by the author have been discussed.

In previous communications (1, 3, 5, 6) it has been shown that mixtures of proteins, peptides and amino acids in amounts ranging from some milligrams to as much as 10–30 grams can be effectively fractionated in a single operation by electrophoresis using cellulose columns. Procedures were also introduced for improving the separation efficiency in zone electrophoresis. In the present paper additional aspects of zone electrophoresis have been discussed with particular reference to the properties of the supporting medium.

Sulfomethyl cellulose, synthesized by the author for the first time and used as adsorbent for serum proteins (4), has also been shown to be of great value for chromatographic purification of peptides (6).

2. Porcine posterior pituitary extract has been fractionated by precipitation and extraction followed by chromatography on cellulose ion exchangers and zone electrophoresis (6). Oxytocin, lysine-vasopressin and β-MSH have been isolated. The examples given demonstrate the potentialities of the methods for fractionation of complex natural mixtures of amino acids, peptides and proteins.

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It is a pleasure for me to express my deep gratitude to Professor A. Tiselius for his great interest in all parts of my work, for his invaluable encouragement and many suggestions in frequent discussions. I am also indebted to my former teacher, Professor A. Fredga, who directed my interest to organic substances of natural origin.

The biological potency of oxytocin and vasopressin has been assayed at the State Pharmaceutical Laboratory, Stockholm, under supervision of Professor H. Rydin and Dr. Ada Elmqvist. I wish to thank them cordially for their indispensible help.

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REFERENCES

1. Porath, J., Biochim. et Biophys. Acta, *22*, 151 (1956).
2. Porath, J., Nature, *175*, 478 (1955).
3. Porath, J., Arkiv Kemi, *10*, No. 38 (1956).
4. Porath, J., Arkiv Kemi, *11*, No. 11 (1957).
5. Porath, J., Arkiv Kemi, *11*, No. 18 (1957).
6. Porath, J., Arkiv Kemi, *11*, No. 28 (1957).
7. Porath, J., Roos, P., Landgrebe, F. W., and Mitchell, G. M., Biochim. et Biophys. Acta, *17*, 598 (1955).
8. Svensson, H., Arkiv Kemi Mineral. Geol. 22A, No. 10 (1946).
9. Tiselius, A., and Flodin, P., Advances in Protein Chemistry, Vol. VIII, 461 (1954).
10. Kunkel, H., *in* Glick, Methods in Biochemical Analysis, Vol. I. Interscience Publ., New York, 1954, p. 163.
11. Brattsten, I., Continuous Zone Electrophoresis by Crossed Velocity Fields in a Supporting Medium. Diss. Uppsala 1955.
12. Tiselius, A., and Laurell, H., Advances in Enzymology, in press.
13. Lederer, M., Introduction to Paper Electrophoresis. Elsevier Publ. Co., Amsterdam, 1955.
14. McDonald, H. J., Ionography. Year Book Publ., Inc., Chicago, 1955.
15. Durrum, E., *in* Block, Durrum, and Zweig, A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, Inc. Publ., New York, 1955, p. 163.
16. Kolin, A., J. Chem. Phys., *22*, 1628 (1954).
17. Bracke, M. K., Arch. Biochem. Biophys., *55*, 175 (1955).
18. Sorof, S., Ott, M. G., and Young, E. M., Arch. Biochem. Biophys., *57*, 140 (1955).
19. Svensson, H., and Valmet, E., "Science Tools", LKB-Instrument J., *2*, p. 11 (1955) (LKB-Produkter Fabriks AB. Box 12035, Stockholm 12, Sweden).
20. Kirkwood, J. G., J. Chem. Phys., *9*, 878 (1941).
21. Cann, J. R., Kirkwood, J. G., Brown, R. A., and Plescia, O. J., J. Biol. Chem., *77*, 1603 (1949).
22. Haglund, H., and Tiselius, A., Acta Chem. Scand., *4*, 957 (1950).
23. Porath, J., Acta Chem. Scand., *8*, 1813 (1954).
24. Sorof, S., and Porath, J., to be published.
25. Smithies, O., Biochem. J., *61*, 629 (1955).
26. Flodin, P., and Kupke, D. W. Biochim. et Biophys. Acta, *21*, 368 (1956).
27. Gordon, A. H., Biochem. J., *63*, 28 P (1956).
28. Kunkel, H., and Slater, R. J., J. Clin. Invest., *31*, 1677 (1952).
29. Flodin, P., and Porath, J., Biochim. et Biophys. Acta, *13*, 175 (1954).
30. Gedin, H. I., and Porath, J., Biochim. et Biophys. Acta, to be published.
31. Consdon, R., Gordon, A. H., and Martin, A. J. P., Biochem. J., *41*, 590 (1947).

32. Porath, J., Arkiv Kemi, *7*, No. 57 (1954).
33. Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A. B., J.A.C.S., *73*, 2969 (1951).
34. Peterson, E. A., and Sober, H. A., J.A.C.S., *78*, 751 (1956).
35. Savage, A. B., Young, A. E., and Maasberg, A. T. *in* High Polymers, Vol. V, 2nd ed., p. 886. Interscience Publ. Inc., New York, 1954.
36. Scharrer, E., and Scharrer, B., *in* Recent Progress in Hormone Research, Vol. X, 183 (1954).
37. van Dyke, H. B., Chow, B. F., Greep, R. O., and Rothen, A., J. Pharmacol. Exptl. Therap., *74*, 190 (1942).
38. Acher, R., Chauvet, J., and Olivry, G., Biochim. et Biophys. Acta, *22*, 421 (1956).
39. Kiessling, H., and Elmqvist, A., to be published.
40. Landgrebe, F. W., and Mitchell, G. M., Quart. J. Exptl. Physiol., *39*, 11 (1954).